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14. ABSTRACT

We propose that the respiratory symptoms of subjects with GWI is caused in part by enhanced airway sensitivity to irritants, mediated primarily by upregulated or sensitized TRP channels in the airway. We have now shown that transformed and primary airway epithelial cells express functional TRPV1. More recently we found that these cells also express another important irritant receptor, namely TRPA1. Activation of either TRPV1 or TRPA1 results in increased expression of both the inflammatory cytokine IL-8 and the neurotrophin NGF in airway epithelial cells. We have further shown that NGF as well as bradykinin and CCL5 can sensitize TRPV1, lower the activation threshold, and enhance the inflammatory consequences of agonist stimulation. The combination of sensitized TRPV1 channels that have a lower threshold for activation from common irritants combined with TRP activation-mediated synthesis of NGF leading to further sensitization of sensory afferents and epithelial cells suggests a mechanism by which non-specific airway sensitivity may be initiated and sustained in a self-perpetuating manner.

15. SUBJECT TERMS

Enhanced airway sensitivity, transient receptor potential, TRPV1, TRPA1, neurotrophin, bradykinin, airway epithelial cells

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INTRODUCTION:

We propose that the increased respiratory symptoms in Gulf War Illness (GWI) occur as a consequence of enhanced airway sensitivity – a diagnosis that may not be detected by standard classification or pulmonary function testing. Our hypothesis is that the underlying basis for the respiratory symptoms of subjects with GWVI is enhanced airway sensitivity to irritants, mediated primarily by upregulated or sensitized TRPV1 channels. To pursue this hypothesis we will assess the relationship between expression and function of TRPV1 channels on airway epithelial cells and sensitivity to capsaicin inhalation challenge. We will also measure the levels of mediators involved in neurogenic responses in bronchoalveolar fluid. We will then use an ex vivo and in vitro approach to assess the mechanistic implications of the in vivo findings. We will analyze how airway epithelial cell TRPV1 upregulation or sensitization may promote the self-perpetuating increase in airway sensitivity, and assess the expression of neurotrophin, bradykinin, tachykinin, and cannabinoid receptors on the brushing airway epithelial cells at the mRNA and protein level. These interactions will be explored by overexpressing, knocking down, or inhibiting selected receptors and analyzing the impact on TRPV1 upregulation or sensitization.

BODY:

The statement of work included seven tasks. Our progress to date is summarized by task below.

***Task #1: Subject recruitment. Subjects with GWI-R as well as matched controls and chronic cough controls will be recruited and screened for eligibility including performing a history and physical, filling out detailed questionnaires, allergy skin testing, screening spirometry and a methacholine inhalation challenge*.**

Subjects with Gulf War Illness and respiratory symtoms (GWI-R) as well as appropriate controls are to be recruited for the studies covered in the subsequent tasks. While we have made substantial effort and progress in this task, we are only now actually beginning to recruit.

We succeeded in obtaining local IRB, Office of Research Protections (ORP) U.S. Army Medical Research and Materiel Command (USAMRMC), and VA R&D approval for our protocols. When we finally had received all of these approvals several months ago, we began to try to recruit subjects through VA nurse practitioners who screen patients for Gulf War Illness; however, we have not been able to enroll any subjects from this strategy to date.

Because of a lack of subjects from this approach, we decided to use the Persian Gulf Registry (PGR) as a recruiting tool. After discussing this with Dr. Han Kang (Director Environmental Epidemiology Service, Department of Veterans Affairs, VACO), our proposal was reviewed and approved by the VACO and the Austin Information Technology Center. We then had to gain approval from the IRB and VA R&D for the change in recruiting plan, and this was finally obtained as of 6/27/10.

We have been in communication with Dr. Jennifer Javors, the Environmental Health Clinician at the San Diego VA, who will assist us in this effort. We are sending out our IRB approved flyer and cover letter to all Veterans who have registered in the PGR and live within a ~100 mile radius of the San Diego VA. The flyer explains the study and requests that interested parties call us to schedule an appointment. We believe that this will be a productive mechanism to recruit subjects and expect to soon be enrolling subjects.

Task #2: Determine whether GWI-R subjects demonstrate increased airway sensitivity to capsaicin challenge. At least one month after the methacholine inhalation challenge subjects will undergo standardized capsaicin inhalation challenge.

Progress on this task awaits recruitment of the subjects described above. We have, however, obtained the pharmaceutical grade capsaicin required for the capsaicin challenges, and have all the necessary equipment to proceed with the challenges once the subjects have been recruited.

Task #3: Determine whether airway bronchial epithelial cells from GWI-R subjects demonstrate increased airway sensitivity to capsaicin challenge. At least one month following the capsaicin inhalation, subjects will return for bronchoscopy. Bronchoscopy will be performed in the VA Research Bronchoscopy Core facility. Airway epithelial cells from the subjects will be recovered by bronchial brushings. These bronchial epithelial cells will be stimulated with capsaicinoid agonists then gene expression and calcium flux outcomes determined.

The ex vivo studies that will be performed on samples from subjects enrolled in this study require substantial technical expertise. Therefore, we have devoted considerable effort to preparing for this important task.

To make sure that we can recover the bronchial epithelial cells in sufficient number to perform the required experiments, we have initiated a separate pilot project to obtain bronchial brushing cells from patients undergoing bronchoscopy for clinical indications. We are using these samples to optimize the handling of brushing samples to obtain the largest number of viable bronchial epithelial cells. Once we have satisfied ourselves that we have optimized the processing of the brushing samples, we will use the bronchial epithelial cells for challenges with capsaicinoid ligand.

The bronchial epithelial cells obtained from study subjects will be used to assess gene expression and calcium flux in response to capsaicinoid agonists. Gene expression will be assessed primarily by quantitative RT-PCR. We have developed highly sensitive real-time quantitative RT-PCR methods to measure gene expression at the mRNA level. We have detected increased expression of inflammatory cytokines such as IL-8 as well as the neutrophin NGF mRNA in response to capsaicinoid stimulation. We have also designed additional primers and probes for this purpose, including IL-1β, TNFα, IL-6, and a variety of chemokines including CCL11 (eotaxin) and CCL5 (RANTES). We are confident that we are ready to detect even small changes in gene expression in the bronchial epithelial cell samples from subjects.

We have previously measured intracellular Ca2+ flux as an overall change in detached cell populations stimulated with ligand. In order to optimize our ability to measure Ca2+ flux in bronchial epithelial cells from subject samples, we have developed the ability to measure changes in Ca2+ in individual cells on a microscope slide by advanced microscopy. using a Zeiss Axio Observer Z1 inverted microscope. We began by loading BEAS-2B and primary human bronchial epithelial cells, grown on glass slides, with Fura-2AM. Following

capsaicin challenge, we observed a weak increase in intracellular calcium detected and quantified in single cells. To improve the strength of this signal we have optimized the loading of the airway epithelial cells. Using Influx Pinocytic Cell Loading Reagent from Molecular Probes to load Fura-2, we have successfully detected changes in calcium flux in response to stimulation with bradykinin and capsaicinoid ligands in both BEAS-2B and normal human bronchial epithelial cells.

Following a report that different novel variants of TRPM8 are found in the airway epithelial cell in comparison to other cells types [1], we assessed whether airway epithelial cells express variants of TRPV1. Four variants of TRPV1 have been reported in the literature [2]. We designed specific primer/probe sets to identify which isoforms of TRPV1 are expressed in airway epithelial cells. All variants share a common coding region from exon 6 to 19 but differed in their 3' exons. To ensure that the region from exon 6 to 19 is fully expressed in airway epithelial cells, we designed 5 sets of primers which span the region, finding that indeed exon 6 to 19 of TRPV1 is transcribed in airway epithelial cells. Variant 1 is composed of exons 1, 2, 5b and then 6 to 19; variant 2 is composed of exons 3, 5b and the common region exon 6 to 19; variant 3 is composed of exons 5a and the common region exon 6 to 19; and finally variant 4 is composed of exons 4, 5b and the common region exon 6 to 19. We detected exon 3, exon 4 and exon 5b, suggesting that BEAS2B cells express TRPV1 variants 2 and 4. This is important novel information, and we will need to confirm this in the bronchial brushing cells.

Importantly, new literature has suggested that a related transient receptor channel, TRPA1, is a key sensor of irritant stimuli and may be important in airway disease [3, 4]. Moreover, TRPV1 and TRPA1 are triggered by many of the same agonists. We therefore asked whether TRPA1 is also expressed in human airway epithelial cells. Using RT-PCR, we found that the entire coding region of TRPA1 is expressed in BEAS-2B and normal human airway epithelial cells. Furthermore, we showed that activation of normal human bronchial epithelial cell TRPA1 with cinnamaldehyde resulted in increases in the expression of both IL-8 and NGF. Ongoing experiments are looking at whether TRPA1 activation has a synergistic effect on the consequences of TRPV1 activation in airway epithelial cells. Based on this, we plan to examine TRPA1 expression in the GWI-R subjects compared to controls, and assess the functional responses to TRPA1 agonists, such as allyl isothiocyanate and cinnamaldehyde.

Task #4: Determine whether the bronchoalveolar lavage (BAL) fluid of subjects with GWI-R contain increased levels of mediators involved in neurogenic responses. BAL fluid performed during the bronchoscopy will be used to measure neurotrophins, tachykinins, and tissue kallikrein activity. As a control, cytokines involved in conventional Th1 and Th2 inflammation will be measured. The cellular profile in the BAL fluid will be

determined.

We have developed the assays required for this aim. In particular, we have developed ELISA and immunoblotting assays to measure cytokine and mediator levels in BAL fluid. We have also developed sensitive chromogenic assays for tissue kallikrein activity that detect this activity in BAL fluid.

Task #5: Determine whether the airway epithelial cells of subjects with GWI-R show evidence of remodeling. Airway epithelial cells from task #3 above as well as bronchial biopsies obtained during the bronchoscopy procedure will be used to further characterize epithelial remodeling. Expression of mRNA for TRPV1, bradykinin receptors, neurotrophin receptors, tachykinin receptors, and cannabinoid receptors will be measured by quantitative real-time RT-PCR in the bronchial brushing cells. We will also identify these receptors by immunofluorescence staining of cytospin slides made with the brushing cells. Immunofluorescence staining of TRPV1 in the bronchial biopsies will be colocalized with neural and epithelial cell markers. If TRPV1 expression is upregulated, the impact will be modeled by overexpressing TRPV1 in normal human bronchial epithelial cells.

As detailed above, we have developed sensitive real-time RT-PCR assays for mRNA transcripts of these genes, including TRPV1, TRPA1, the bradykinin B2 and B1 receptors, the entire range of neurotrophin receptors, and the cannabinoid receptor, CNR2. We still need to design and test primers for the tachykinin receptors.

We have also worked to detect TRPV1 and bradykinin receptors by immunofluorescence. We were able to detect TRPV1 and B2 bradykinin receptors on airway epithelial cells by standard immunofluorescence techniques using commercial antibodies. The B1 bradykinin receptor, however, was difficult to detect in these cells. We have now obtained additional antibodies to the B1 bradykinin receptor, including a custom bradykinin B1 receptor antibody from Dr. Werner Muller-Esterl, University of Frankfurt Medical School, Germany. Using these antibodies, we can now detect B1 bradykinin receptors by immunoblotting and will soon attempt to detect them by immunofluorescence as well.

Task #6: Determine whether increased expression of neurotrophin, bradykinin, or substance P receptors enhance TRPV1 upregulation or sensitization in airway epithelial cells. Receptors found to be expressed at increased levels in vivo will be overexpressed in cultured primary NHBE cells in vitro. The effect of overexpressing these receptors on the ability of its ligand to induce TRPV1 upregulation or sensitization will then be determined.

We demonstrated that pretreatment of airway epithelial cells with bradykinin, NGF, or CCL5 resulted in enhanced TRPV1 signaling and gene expression. To pursue this observation, we want to examine the regulation and phosphorylation of TRPV1. As demonstrated above, we have developed techniques that will allow us to measure TRPV1 expression at the mRNA and protein levels.

We are also working to develop techniques to assess phosphorylation of TRPV1 in bronchial epithelial cells. One approach that we are exploring is to use an immunoprecipitation/pulldown technique followed by immunoblotting of the TRPV1 for phosphorylated residues. We constructed a GFP-TRPV1 fusion protein that we have transfected into airway epithelial cells. We are currently analyzing the pulldown results using our anti-TRPV1 antibodies. Once we show that we can pulldown the GFP-TRPV1 protein, we can begin to work on the immunoblotting to detect phosphorylation following treatment of the cells with bradykinin, NGF, or CCL5. Ultimately, we will also use these techniques to study bronchial epithelial cells from the GWI-R subjects.

Task #7: Determine whether knockdown of neurotrophin, bradykinin, or substance P receptors or inhibition of key signaling pathways attenuate TRPV1 upregulation or sensitization in airway epithelial cells. The receptors upregulated in task #6 will also be knocked down using siRNA. The effect of knocking each of these receptors down on TRPV1 function will then be determined. In addition we will inhibit selected signaling pathways thought to be involved in TRPV1 regulation using pharmacologic inhibitors followed by siRNA mediated knockdown. The impact of inhibiting these pathways on TRPV1 upregulation, sensitization and function will be assessed.

We have made an important advance in our technique that will be used for these experiments. Knockdown of receptors or signaling molecules was to be accomplished by transfection with specific siRNA. Because of the difficulty transfecting primary airway epithelial cells, we had to use Amaxa nucleofection, a technique that both requires large numbers of cells and results in the death of many of the cells. We have now developed conditions to efficiently transfect primary bronchial epithelial cells with a specialized lipid-based transfection reagent (HiPerFect, Qiagen). We now achieve efficient transfection of primary human bronchial epithelial cells with little cell death. This should allow us to conduct the studies for this task much more efficiently.

KEY RESEARCH ACCOMPLISHMENTS:

- Primary and transformed human airway epithelial cells express functional TRPV1
- Airway epithelial cells express the TRPV1 variants 2 and 4
- Airway epithelial cells express functional TRPA1
- Activation of TRPV1 induces IL-8 and NGF expression in airway epithelial cells
- Activation of TRPA1 induces IL-8 and NGF expression in airway epithelial cells
- Inflammatory mediators, including bradykinin, NGF and CCL5, sensitize TRPV1 in airway epithelial cells
- Key technical advances have been accomplished, including measurement of gene expression, intracellular calcium flux in individual bronchial epithelial cells, immunofluorescent detection of TRPV1 and bradykinin receptors, and transfection of primary bronchial epithelial cells

REPORTABLE OUTCOMES:

None.

CONCLUSION:

We propose that the respiratory symptoms of subjects with GWI is caused in part by enhanced airway sensitivity to irritants, mediated primarily by upregulated or sensitized TRP channels in the airway. We have now shown that transformed and primary airway epithelial cells express functional TRPV1. More recently we found that these cells also express another, potentially important, irritant receptor – TRPA1. Activation of either TRPV1 or TRPA1 results in increased expression of both the inflammatory cytokine IL-8 and the neurotrophin NGF in airway epithelial cells. Furthermore, we have shown that NGF as well as bradykinin and CCL5 can sensitize TRPV1 and enhance the inflammatory consequences of agonist stimulation. The combination of sensitized TRPV1 channels that have a lower threshold for activation from common irritants combined with TRPV1 activation-mediated synthesis of NGF leading to further sensitization of sensory afferents and epithelial cells suggests a mechanism by which non-specific airway sensitivity may be initiated and sustained in a self-perpetuating manner.

To address this possibility we have proposed to analyze patients with GWI and respiratory symptoms for evidence of enhanced TRPV1-mediated sensitivity. During the first year of this project we have developed essentially all the techniques needed to perform the proposed tasks. In particular, we have developed techniques to measure TRPV1 and TRPA1 expression and function in bronchial epithelial cells, as well as to overexpress and knockdown these ion channels in both transformed and primary airway epithelial cells. We have also cleared all the regulatory hurdles for recruiting the subjects needed for this project. The immediate goal for the near future is to successfully recruit patients with GWI-R utilizing the Persian Gulf Registry database.

Confirmation of our hypothesis would have several important implications. First, it could provide a pathophysiologic explanation for the observed frequency of respiratory complaints in patients suffering from GWI. Secondly, it could provide an objective way to diagnosis GWI-R or measure response to therapy. Finally, it could suggest clear strategies for treating patients with GWI-R.

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APPENDICES:

None.